Elucidation of Mycobacterium abscessus aminoglycoside and capreomycin resistance by targeted deletion of three putative resistance genes

Rominski, Anna ; Selchow, Petra ; Becker, Katja ; Brülle, Juliane K ; Dal Molin, Michael ; Sander, Peter

Abstract: Objectives Mycobacterium abscessus is innately resistant to a variety of drugs thereby limiting therapeutic options. Bacterial resistance to aminoglycosides (AGs) is conferred mainly by AG-modifying enzymes, which often have overlapping activities. Several putative AG-modifying enzymes are encoded in the genome of M. abscessus . The aim of this study was to investigate the molecular basis underlying AG resistance in M. abscessus . Methods M. abscessus deletion mutants deficient in one of three genes potentially involved in AG resistance, aac(2 ') , eis1 and eis2 , were generated by targeted gene inactivation, as were combinatorial double and triple deletion mutants. MICs were determined to study susceptibility to a variety of AG drugs and to capreomycin. Results Deletion of aac(2 ') increased susceptibility of M. abscessus to kanamycin B, tobramycin, dibekacin and gentamicin C. Deletion of eis2 increased susceptibility to capreomycin, hygromycin B, amikacin and kanamycin B. Deletion of eis1 did not affect drug susceptibility. Equally low MICs of apramycin, arbekacin, isepamicin and kanamycin A for WT and mutant strains indicate that these drugs are not inactivated by either AAC(2 ') or Eis enzymes. Conclusions M. abscessus expresses two distinct AG resistance determinants, AAC(2 ') and Eis2, which confer clinically relevant drug resistance.

DOI: https://doi.org/10.1093/jac/dkx125

J Antimicrob Chemother 2017; **72**: 2191–2200
doi:10.1093/jac/dkx125 Advance Access publication 9 May 2017

Journal of Antimicrobial Chemotherapy

Elucidation of *Mycobacterium abscessus* aminoglycoside and capreomycin resistance by targeted deletion of three putative resistance genes

Anna Rominski[1], Petra Selchow[1], Katja Becker[1], Juliane K. Brülle[1], Michael Dal Molin[1] and Peter Sander[1,2]*

[1]*Institut für Medizinische Mikrobiologie, Universität Zürich, Gloriastrasse 30/32, 8006 Zürich, Switzerland;* [2]*Nationales Zentrum für Mykobakterien, Gloriastrasse 30/32, 8006 Zürich, Switzerland*

*Corresponding author. Institut für Medizinische Mikrobiologie, Universität Zürich, Gloriastrasse 30/32, 8006 Zürich, Switzerland. Tel: +41-44-634-26-84; Fax: +41-44-634-49-06; E-mail: psander@imm.uzh.ch

Received 21 December 2016; returned 16 February 2017; revised 10 March 2017; accepted 3 April 2017

Objectives: *Mycobacterium abscessus* is innately resistant to a variety of drugs thereby limiting therapeutic options. Bacterial resistance to aminoglycosides (AGs) is conferred mainly by AG-modifying enzymes, which often have overlapping activities. Several putative AG-modifying enzymes are encoded in the genome of *M. abscessus*. The aim of this study was to investigate the molecular basis underlying AG resistance in *M. abscessus*.

Methods: *M. abscessus* deletion mutants deficient in one of three genes potentially involved in AG resistance, *aac(2′)*, *eis1* and *eis2*, were generated by targeted gene inactivation, as were combinatorial double and triple deletion mutants. MICs were determined to study susceptibility to a variety of AG drugs and to capreomycin.

Results: Deletion of *aac(2′)* increased susceptibility of *M. abscessus* to kanamycin B, tobramycin, dibekacin and gentamicin C. Deletion of *eis2* increased susceptibility to capreomycin, hygromycin B, amikacin and kanamycin B. Deletion of *eis1* did not affect drug susceptibility. Equally low MICs of apramycin, arbekacin, isepamicin and kanamycin A for WT and mutant strains indicate that these drugs are not inactivated by either AAC(2′) or Eis enzymes.

Conclusions: *M. abscessus* expresses two distinct AG resistance determinants, AAC(2′) and Eis2, which confer clinically relevant drug resistance.

Introduction

Mycobacterium abscessus is a rapid growing mycobacterium of increasing medical importance. This emerging pathogen causes bronchopulmonary infections in individuals with cystic fibrosis[1] and chronic pulmonary disease, such as pneumoconiosis and bronchiectasis.[2] It also causes severe infections following surgery, transplantation, tattooing and mesotherapy.[3–8] Treatment of an *M. abscessus* infection is difficult due to the bacteria's high degree of intrinsic resistance to chemotherapeutic agents.[9] The pathogen is naturally resistant to many major classes of antibiotics used for the treatment of Gram-positive and Gram-negative bacterial infections, such as β-lactams, aminoglycosides (AGs) (kanamycin B, gentamicin C) and macrolides (erythromycin). In addition, it is also resistant to first-line TB drugs, for instance isoniazid and rifampicin. *M. abscessus* has been called an antibiotic nightmare since treatment options against *M. abscessus* infection are more limited than for *Mycobacterium tuberculosis* infection.[10] While no standard treatment recommendations for pulmonary *M. abscessus* infections have yet been established, current guidelines propose administration of an oral macrolide (clarithromycin or azithromycin) for clinical isolates susceptible to macrolides and the intravenous AG amikacin in combination with a parenteral β-lactam antibiotic, cefoxitin or imipenem.[11] AGs and macrolides inhibit protein biosynthesis by binding to the small and large ribosomal subunit, respectively. Clinically acquired pan-AG and pan-macrolide resistance has been attributed to mutations in ribosomal RNA genes *rrs* and *rrl*, coding for 16S and 23S rRNA, respectively. Owing to the presence of a single ribosomal RNA (*rrn*) operon, corresponding resistance mutations have readily been observed in *M. abscessus*.[12–14] However, distinct mechanisms have been proposed to be responsible for innate AG resistance in *M. abscessus*.[9,15]

AG antibiotics form a group of hydrophilic molecules, consisting of a characteristic, central aminocyclitol linked to one or more amino sugars by pseudoglycosidic bond(s). They inhibit prokaryotic protein biosynthesis by binding to the A-site of the 16S rRNA. AGs are active against a wide range of aerobic Gram-negative bacilli, staphylococci and mycobacteria. Resistance to AGs is due to low uptake, increased efflux, target modification

or enzyme-mediated drug modification. Originally isolated from microorganisms (mainly *Streptomyces* spp.), semi-synthetic derivatives of AGs have been generated to improve the pharmacological properties of the drugs and to overcome bacterial resistance mechanisms.[16] The semi-synthetic drug amikacin is a cornerstone of *M. abscessus* infection therapy, whereas other AGs are not frequently used. *M. abscessus* genome annotation suggests the presence of various AG-modifying enzymes such as AG phosphotransferases, AG nucleotidyltransferases and AG acetyltransferases (AACs).[17] The individual contribution of these genes to *M. abscessus* AG susceptibility cannot be predicted reliably due to overlapping specificities.

Elucidation of gene functions, drug target and host–pathogen interaction heavily relies on the generation of isogenic mutants. Usually, antibiotic resistance markers are used for the primary selection of transformants. Tools for genetic manipulation of mycobacteria in general, and *M. tuberculosis* in particular, have been developed during the past two or three decades.[18] In contrast, genetic manipulation of *M. abscessus* has been reported to be very difficult and attempts have often been unsuccessful,[19–21] although some progress has been made.[22–25] We recently developed genetic tools for *M. abscessus*[26] and have now exploited them for elucidating intrinsic AG resistance mechanisms in the *M. abscessus* ATCC 19977 type strain. We particularly addressed the role of putative AACs in *M. abscessus* AG and capreomycin resistance. We generated single, double and triple mutants of *M. abscessus* by targeted deletion of three genes potentially involved in AG resistance and characterized those mutants by phenotypic drug susceptibility testing. Interestingly, a double mutant proved to be susceptible to a wide variety of AGs and to the peptide antibiotic capreomycin.

Materials and methods

Bacterial strains and media

The *Escherichia coli* laboratory strain XL1-Blue MRF′ (Stratagene, Switzerland) and ER2925 *dam*$^{-}$, *dcm*$^{-}$ were used for cloning and propagation of plasmids. The strains were grown in LB medium containing one of the antibiotics: ampicillin (120 mg/L), kanamycin A (50 mg/L), apramycin (50 mg/L) or hygromycin B (100 mg/L). Antibiotics (Sigma–Aldrich, Switzerland) were dissolved in water according to the manufacturer's recommendations and stored as a stock solution until usage.

M. abscessus ATCC 19977 was grown in Middlebrook 7H9-OADC-Tween 80 or LB containing one of the antibiotics, if required: kanamycin A (50 mg/L), apramycin (50 mg/L) or isoniazid (32 mg/L). For preparation of electrocompetent cells two cell culture flasks each containing 200 mL of 7H9-OADC-Tween 80 were inoculated 1:100, incubated at 37°C and gently shaken daily. When OD_{600} reached 0.4–0.8, cultures were set on ice for 90 min. Bacteria were harvested by centrifugation and repeatedly resuspended in ice-cold glycerol (10% v/v) while gradually reducing the volume.[27] Finally, bacteria were resuspended in 2 mL of glycerol (10% v/v) and either frozen in liquid nitrogen or directly used for electroporation, as we recently described.[26] Genomic DNA was isolated by phenol/chloroform/isoamyl alcohol extraction, as described previously.[28]

Generation of vectors

The apramycin resistance cassette from plasmid pSET152[29] was PCR amplified (primers Apr_F and Apr_R) (see Table S1, available as Supplementary data at *JAC* Online, for list of primers) and cloned as a 1 kbp SpeI fragment into SpeI-digested plasmid pMV361[30] to result in plasmid pMV361-*apr*. An *M. tuberculosis katG* fragment (2.8 kbp) was PCR amplified (primers KatG_F and KatG_R) and cloned into pGEM-T-easy to result in pGEM-T-*katG*. Subsequently, the 2.8 kbp SwaI-*katG*-HpaI fragment was cloned into HpaI-linearized pMV361-*apr* to result in pMV361-*apr-katG*. Removal of mycobacteriophage *attP/int* from plasmid pMV361-*apr-katG* by XbaI excision and self-ligation of the vector backbone resulted in the prototype suicide vector pSE-*apr-katG* into which flanking regions of the target genes were cloned.

Disruption of MAB_4395 [aac(2′)] in M. abscessus

A 1.6 kbp NdeI/NheI fragment of *M. abscessus* ATCC 19977 from position 4477184 to 4478794 comprising the 5′ flanking sequence and a 1.5 kbp NheI/MluI fragment from position 4479125 to 4480674 comprising the 3′ flanking sequence of *MAB_4395* [*aac(2′)*] were PCR amplified (primers 4395_UP_F, 4395_UP_R and 4395_DW_F, 4395_DW_R) using genomic DNA from *M. abscessus* ATCC 19977. Subsequently, fragments were ligated stepwise into saving vector pMCS5 with respective enzymes resulting in subcloning vector pMCS5-*MAB_4395*. The region comprising 5′ and 3′ flanking sequences of *aac(2′)* was cut out with StuI and cloned into HpaI-linearized pSE-*apr-katG* vector to result in knockout vector pSE-Δ*aac(2′)*. The *aac(2′)* allele was deleted from the *M. abscessus* ATCC 19977 chromosome using apramycin for positive and isoniazid for negative selection. Deletion of *aac(2′)* was confirmed by Southern blot analysis of Van91I-digested genomic DNA with a 0.3 kbp 5′ *aac(2′)* probe amplified with primers P_4395_F and P_4395_R. A 2.2 kbp fragment from *M. abscessus* ATCC 19977 from position 4477131 to 4479338 spanning the entire *aac(2′)* gene was amplified with primers C_4395_F and C_4395_R and cloned into HindIII-digested pMV361 vector to result in pMV361-*aac(2′)* complementation vector. *M. abscessus* Δ*aac(2′)* was transformed with pMV361-*aac(2′)* and control vector pMV361, respectively. Genetic complementation was confirmed by Southern blot analysis with the same 5′ *aac(2′)* probe.

Disruption of MAB_4124 (eis1) in M. abscessus

A 1.6 kbp ApaI/NdeI fragment of *M. abscessus* ATCC 19977 from position 4187416 to 4189052 comprising the 5′ flanking sequence and a 1.7 kbp NdeI/NheI fragment from position 4189857 to 4191580 comprising the 3′ flanking sequence of *MAB_4124* (*eis1*) were PCR amplified (primers 4124_UP_F, 4124_UP_R and 4124_DW_F, 4124_DW_R) using genomic DNA from *M. abscessus* ATCC 19977. Fragments were stepwise cloned with corresponding enzymes into saving vectors and subsequently into NotI/XbaI-digested vector pSE-*apr-katG* to result in knockout vector pSE-Δ*eis1*. The *eis1* allele was deleted from the *M. abscessus* ATCC 19977 chromosome using apramycin (positive) and isoniazid (negative) selection. Deletion was confirmed by Southern blot analysis using Van91I-digested genomic DNA and a 0.3 kbp 5′ *eis1* fragment amplified with primers P_4124_F and P_4124_R.

Disruption of MAB_4532c (eis2) in M. abscessus

A 1.8 kbp HpaI/Pfl23II fragment of *M. abscessus* ATCC 19977 from position 4612843 to 4614693 comprising the 5′ flanking sequence and a 1.5 kbp PacI/Pfl23II fragment from position 4615391 to 4616869 comprising the 3′ flanking sequence of *MAB_4532c* (*eis2*) were PCR amplified (primers 4532c_UP_F, 4532c_UP_R and 4532c_DW_F, 4532c_DW_R) using genomic DNA from *M. abscessus* ATCC 19977. Fragments were stepwise cloned into vector pSE-*apr-katG* resulting in knockout vector pSE-Δ*eis2*. The *eis2* allele was deleted from the *M. abscessus* ATCC 19977 chromosome using apramycin (positive) and isoniazid (negative) selection. Deletion was confirmed by Southern blot analysis with Van91I-digested genomic DNA and a 0.3 kbp 5′ *eis2* probe amplified with primers P_4532c_F and P_4532c_R. For complementation a 1.3 kbp fragment from *M. abscessus* ATCC 19977 (position 4614320–4615651) spanning the entire *eis2* gene was PCR amplified (primers C_4532c_F, C_4532c_R) and cloned via HindIII into pJB-*apr* vector to result in pJB-*apr-eis2* complementation vector. *M. abscessus* Δ*eis2* was

transformed with pJB-*apr-eis2* and control vector pJB-*apr*, respectively. Genetic complementation was confirmed by Southern blot analysis.

MIC assays

Kanamycin B, tobramycin, dibekacin, arbekacin, gentamicin C [C1 (<45%), C2 (<35%) and C1a (<30%)], isepamicin, amikacin, kanamycin A, apramycin, streptomycin, hygromycin B and capreomycin were bought from Sigma–Aldrich. Antibiotics were dissolved in water according to the manufacturer's recommendations, were filter sterilized, aliquoted into stock solutions and stored at −20°C. MIC determination for *M. abscessus* strains was done according to CLSI guidelines[31] and basically as we previously described.[26]

Results

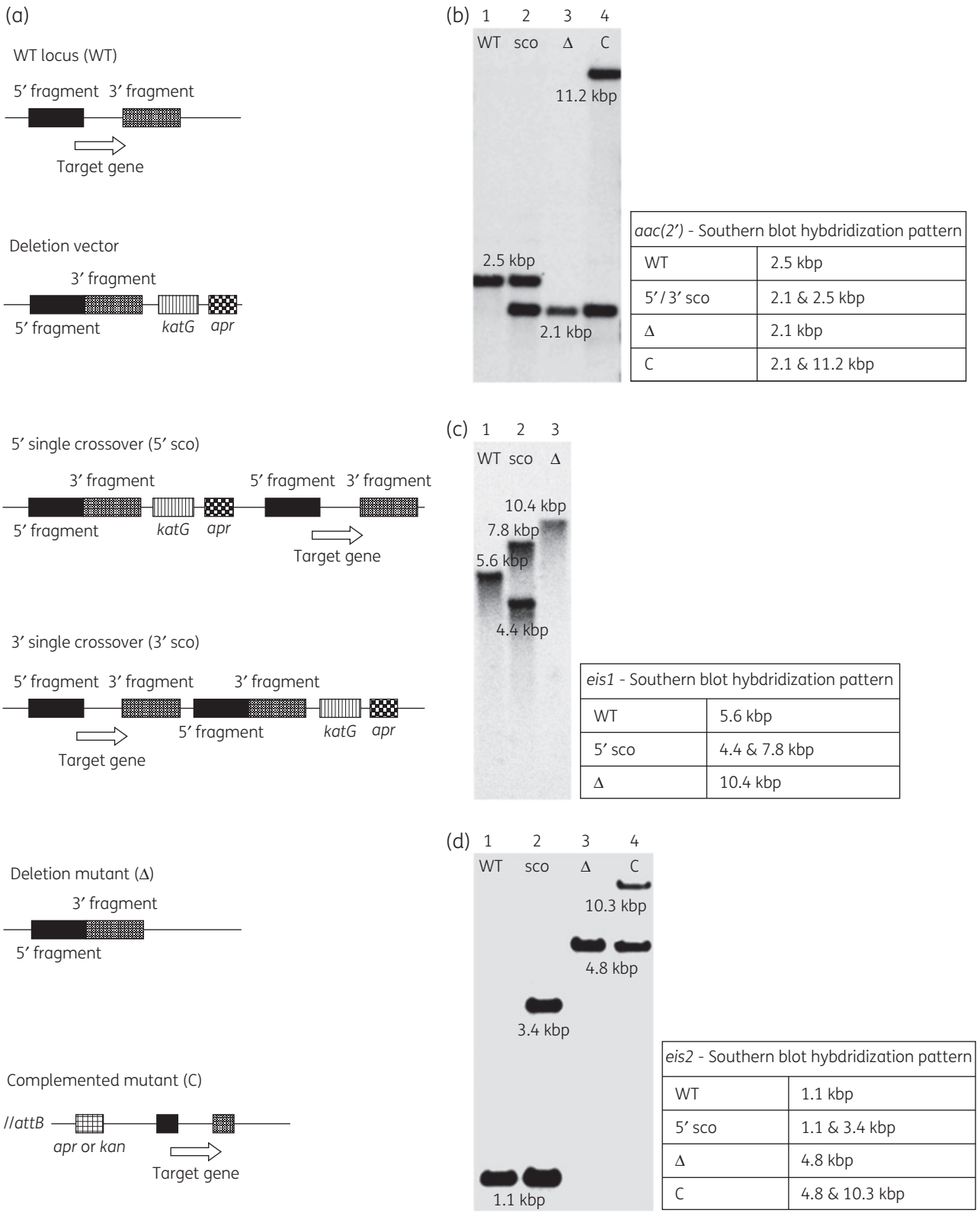
Targeted inactivation of putative AG resistance genes of M. abscessus

Genome annotation,[17] genome analysis and AG drug susceptibility testing[15] suggest the presence of several putative AACs in *M. abscessus*. ORF *MAB_4395* is annotated as a putative AG 2′-*N*-acetyltransferase [*aac(2′)*],[17] while *MAB_4124* and *MAB_4532c* show homology to *eis*. Eis (enhanced intracellular survival) proteins are found in a variety of mycobacterial and non-mycobacterial species.[32,33] Overexpression of Eis (Rv2416c) confers increased kanamycin resistance on *M. tuberculosis*.[34] *M. abscessus MAB_4124* is the closest homologue of *M. tuberculosis Rv2416c* (33% identity), which we therefore name *eis1*. Interestingly, a second Eis homologue encoded by *MAB_4532c* is present in the *M. abscessus* genome. In a phylogenetic tree constructed from 29 Eis homologues,[33] MAB_4532c (which we name Eis2) is clustered with Eis homologues from the non-mycobacterial clade instead of the mycobacterial group, e.g. *M. abscessus* Eis2 shows 23% identity to *Anabaena variabilis* Eis. We addressed the role of the three acetyltransferases in *M. abscessus* AG resistance by engineering and characterizing corresponding unmarked deletion mutants in the *M. abscessus* ATCC 19977 type strain.

Flanking fragments of the target genes *aac(2′)*, *eis1* and *eis2* were cloned into plasmid pSE-*apr-katG* to result in suicide vectors pSE-Δ*aac*, pSE-Δ*eis1* and pSE-Δ*eis2*, respectively. These flanking fragments enable homologous recombination of the suicide vector. The apramycin resistance cassette of the vector backbone facilitates selection of single crossover transformants resulting from vector integration by intermolecular homologous recombination at the target locus. Catalase–peroxidase KatG, the activator of the TB prodrug isoniazid,[35] serves as a negative selectable marker since it sensitizes *M. abscessus* to isoniazid. Expression of KatG facilitates screening for deletion mutants resulting from resolving the integration of the suicide vector by a second intramolecular homologous recombination.[26] Electrocompetent *M. abscessus* were transformed and apramycin selection and Southern blot analysis were applied to identify single crossover transformants. Subsequently, isoniazid counterselection and Southern blot analysis were applied for the identification of *M. abscessus* Δ*aac(2′)*, *M. abscessus* Δ*eis1* and *M. abscessus* Δ*eis2* mutants (Figure 1). Deletion mutants showing a phenotype [*M. abscessus* Δ*aac(2′)* and *M. abscessus* Δ*eis2*] were transformed with single copy, i.e. pMV361-based complementation and control vectors, respectively (see Table 1 for list of strains and Table 2 for list of plasmids). Furthermore, single deletion mutants served as parental strains for construction of double deletion mutants by transformation with a second targeting vector. Double deletion mutants, *M. abscessus* Δ*aac(2′)* Δ*eis1*, *M. abscessus* Δ*aac(2′)* Δ*eis2* and *M. abscessus* Δ*eis1* Δ*eis2*, were confirmed by Southern blot analyses. Finally, by transformation of pSE-Δ*aac(2′)* into *M. abscessus* Δ*eis1* Δ*eis2*, the triple mutant *M. abscessus* Δ*aac(2′)* Δ*eis1* Δ*eis2* was constructed (Figure 2).

Drug susceptibility testing of M. abscessus mutant strains

Drug susceptibility of *M. abscessus* strains towards a variety of structurally similar 4,6-disubstituted AGs (Figure 3) was tested in CAMHB. In addition, drug susceptibility to structurally atypical AGs apramycin, hygromycin B and streptomycin and to the peptide antibiotic capreomycin (also inhibiting protein biosynthesis by interaction with the 16S rRNA A-site) was determined. Growth was judged after 3, 5, 7 and 12 days of incubation and the MICs were determined. The median MIC values on day 5 are shown in Table 3. The MIC values read at all timepoints are shown in Table S2. A variety of mutant phenotypes was observed. Deletion of *aac(2′)* increased susceptibility of *M. abscessus* to kanamycin B (64-fold), tobramycin (32-fold), dibekacin (16-fold) and gentamicin C (4-fold). Deletion of *aac(2′)* did not affect MICs of arbekacin, isepamicin, amikacin, kanamycin A, apramycin, streptomycin, hygromycin B and capreomycin (Table 3). The tobramycin, dibekacin and gentamicin C WT phenotype was restored upon transformation with the complementation vector pMV361-*aac(2′)*, but not by transformation with the backbone control vector pMV361 (Table S2). The presence of a kanamycin resistance cassette [*aph(3′)*] in the backbone of the complementation vector interfered with susceptibility testing for kanamycin A and B and MICs of these compounds are therefore not reported. Deletion of *eis1* did not result in a detectable phenotype; MICs of none of the tested antibiotics were altered. Owing to the absence of a phenotype, no complementation vector was constructed. Deletion of *eis2* increased susceptibility of *M. abscessus* to several AGs. However, the susceptibility pattern of *M. abscessus* Δ*eis2* clearly differed from that of *M. abscessus* Δ*aac(2′)*. Deletion of *eis2* increased susceptibility to kanamycin B (4-fold), amikacin (8-fold), hygromycin B (16-fold) and capreomycin (32-fold). Deletion of *eis2* did not affect susceptibility to tobramycin, dibekacin, arbekacin, gentamicin C, isepamicin, kanamycin A, apramycin and streptomycin. Transformation of the Δ*eis2* mutant with the complementation vector pJB-*apr-eis2*, but not with the vector backbone pJB-*apr*, restored WT MICs of amikacin, hygromycin B and capreomycin (Table S2). The presence of the apramycin resistance cassette in the backbone of the complementation vector interfered with susceptibility testing for kanamycin (A and B), tobramycin, dibekacin, gentamicin C and apramycin, and therefore MIC values of these antibiotics are not reported. Double mutant *M. abscessus* Δ*aac(2′)* Δ*eis1* had the same phenotype as the Δ*aac(2′)* single mutant. Double mutant *M. abscessus* Δ*eis1* Δ*eis2* had the same phenotype as the Δ*eis2* single mutant. The *M. abscessus* Δ*aac(2′)* Δ*eis2* double mutant showed increased susceptibility to kanamycin B, tobramycin, dibekacin, gentamicin C,



aac(2′) - Southern blot hybridization pattern

WT	2.5 kbp
5′ / 3′ sco	2.1 & 2.5 kbp
Δ	2.1 kbp
C	2.1 & 11.2 kbp

eis1 - Southern blot hybridization pattern

WT	5.6 kbp
5′ sco	4.4 & 7.8 kbp
Δ	10.4 kbp

eis2 - Southern blot hybridization pattern

WT	1.1 kbp
5′ sco	1.1 & 3.4 kbp
Δ	4.8 kbp
C	4.8 & 10.3 kbp

Figure 1. Genotypic analyses of *M. abscessus* deletion mutants. (a) General schematic drawing of genotypes and recombination events. (b) Southern blot analysis confirms the deletion of *aac(2′)* (*MAB_4395*) from the genome of *M. abscessus*. Genomic DNA of *M. abscessus* ATCC 19977 (lane 1), *M. abscessus* transformant with pSE-Δ*aac(2′)* targeting vector prior to (lane 2) and after KatG-dependent isoniazid counterselection (lane 3) and after transformation of counterselected mutant with pMV361-*aac(2′)* complementation vector (lane 4) was digested with Van91I and probed with a

amikacin, hygromycin B and capreomycin, whereas MICs of arbekacin, isepamicin, kanamycin A, apramycin and streptomycin were not altered. Thus, the phenotype of the Δ*aac(2′)* Δ*eis2* double mutant is a combination of the phenotype of the single mutants. The phenotype of the triple Δ*aac(2′)* Δ*eis1* Δ*eis2* mutant was similar to the phenotype of the Δ*aac(2′)* Δ*eis2* double mutant. Together, these data indicate a distinct role of *aac(2′)* and of *eis2* in intrinsic AG and capreomycin resistance in *M. abscessus*. Notably, AAC(2′) and Eis2 prevent antibacterial effects of different subsets of AGs. The only exception is kanamycin B, which is modified by both AAC(2′) and Eis2. However, the deletion of *aac(2′)* had a stronger effect on kanamycin B susceptibility than the deletion of *eis2* (64-fold versus 4-fold). AAC(2′) is more influential than Eis2, since the MIC for the double deletion mutant is not further decreased as compared with the *aac(2′)* deletion mutant. Deletion of *eis1* from the genome of the Δ*aac(2′)* Δ*eis2* mutant did not further increase AG susceptibility, indicating a negligible role of Eis1 in *M. abscessus* intrinsic AG resistance, even in an *aac(2′) eis2* double deletion mutant.

Discussion

M. abscessus is a pathogen of increasing medical importance, particularly in individuals with chronic pulmonary disease. The high level of intrinsic resistance to many classes of antibiotics restricts antibiotic therapy.[9] Genome annotation,[17] susceptibility testing and biochemical assays predicted a role for 2′-*N*-acetyltransferase in *M. abscessus* AG resistance.[15] A candidate gene eventually responsible for the increased MIC levels of 2′-amino-AGs, *MAB_4395* [*aac(2′)*] was inactivated by targeted gene deletion. Comparison of WT and mutant strains by phenotypic susceptibility testing demonstrated a decreased MIC for the mutant of a variety of drugs characterized by a 2′-amino group (kanamycin B, tobramycin, dibekacin, gentamicin C). Kanamycin A, which differs from kanamycin B by a single structural feature (2′-OH group instead of a 2′-amino group), has a lower MIC for the WT strain than kanamycin B and its MIC is not further decreased by *aac(2′)* deletion. These data corroborate the hypothesis that *MAB_4395* encodes a functional AG 2′-*N*-acetyltransferase, which renders *M. abscessus*

Table 1. Strains used in this study

Strain	Description	Source
E. coli XL1-Blue MRF'	cloning and propagation of plasmids	Stratagene
E. coli ER2925 *dam*$^{-}$, *dcm*$^{-}$	cloning and propagation of plasmids with methylase susceptible restriction enzymes	New England BioLabs
M. abscessus ATCC 19977	*M. abscessus* type strain	Ripoll *et al.* 2009[17]
M. abscessus Δ*aac(2′)*	*M. abscessus aac(2′)* deletion mutant; derivative of *M. abscessus* ATCC 19977	this study
M. abscessus Δ*eis1*	*M. abscessus eis1* deletion mutant; derivative of *M. abscessus* ATCC 19977	this study
M. abscessus Δ*eis2*	*M. abscessus eis2* deletion mutant; derivative of *M. abscessus* ATCC 19977	this study
M. abscessus Δ*aac(2′)* Δ*eis1*	*M. abscessus aac(2′) eis1* double deletion mutant; derivative of *M. abscessus* Δ*eis1*	this study
M. abscessus Δ*aac(2′)* Δ*eis2*	*M. abscessus aac(2′) eis2* double deletion mutant; derivative of *M. abscessus* Δ*aac(2′)*	this study
M. abscessus Δ*eis1* Δ*eis2*	*M. abscessus eis1 eis2* double deletion mutant; derivative of *M. abscessus* Δ*eis2*	this study
M. abscessus Δ*aac(2′)* Δ*eis1* Δ*eis2*	*M. abscessus aac(2′) eis1 eis2* triple deletion mutant; derivative of *M. abscessus* Δ*eis1* Δ*eis2*	this study
M. abscessus Δ*aac(2′)* pMV361-*aac(2′)*	*M. abscessus aac(2′)* mutant transformed with complementation vector pMV361-*aac(2′)*	this study
M. abscessus Δ*aac(2′)* pMV361	*M. abscessus aac(2′)* mutant transformed with pMV361 control vector	this study
M. abscessus Δ*eis2* pJB-*apr-eis2*	*M. abscessus* Δ*eis2* mutant transformed with complementation vector pJB-*apr-eis2*	this study
M. abscessus Δ*eis2* pJB-*apr*	*M. abscessus* Δ*eis2* mutant transformed with control vector pJB-*apr*	this study

Figure 1. *Continued*

fragment from the 5′ *aac(2′)* flanking region. Based on *M. abscessus* genome annotation and vector sequence, the pattern is consistent with hybridization to a 2.5 kbp fragment of the WT parental strain, to the 2.1 and 2.5 kbp fragments after site-specific homologous recombination (single crossover; sco), to a 2.1 kbp fragment of the *M. abscessus* Δ*aac(2′)* mutant (Δ) and to the 2.1 and 11.2 kbp fragments of the *M. abscessus* Δ*aac(2′)* pMV361-*aac(2′)* complemented mutant strain (C). (c) Southern blot analysis confirms the deletion of *eis1* (*MAB_4124*) from the genome of *M. abscessus*. Genomic DNA of *M. abscessus* ATCC 19977 (lane 1), *M. abscessus* transformant with pSE-Δ*eis1* targeting vector prior to (lane 2) and after KatG-dependent isoniazid counterselection (lane 3) was digested with Van91I and probed with a fragment from the 5′ *eis1* flanking region. Based on *M. abscessus* genome annotation and vector sequence, the pattern is consistent with hybridization to a 5.6 kbp fragment of the WT parental strain, to the 4.4 and 7.8 kbp fragments after site-specific homologous recombination (sco) and to a 10.4 kbp fragment of the *M. abscessus* Δ*eis1* mutant (Δ). (d) Southern blot analysis confirms the deletion of *eis2* (*MAB_4532c*) from the genome of *M. abscessus*. Genomic DNA of *M. abscessus* ATCC 19977 (lane 1), *M. abscessus* transformant with pSE-Δ*eis2* targeting vector prior to (lane 2) and after KatG-dependent isoniazid counterselection (lane 3) and after transformation of counterselected mutant with pJB-*apr-eis2* complementation vector (lane 4) was digested with Van91I and probed with a fragment from the 5′ *eis2* flanking region. Based on *M. abscessus* genome annotation and vector sequence, the pattern is consistent with hybridization to a 1.1 kbp fragment of the WT parental strain, to the 1.1 and 3.4 kbp fragments after site-specific homologous recombination (sco), to a 4.8 kbp fragment of the *M. abscessus* Δ*eis2* mutant (Δ) and to the 4.8 and 10.3 kbp fragments of the *M. abscessus* Δ*eis2* pJB-*apr-eis2* complemented mutant strain (C).

Table 2. Plasmids used in this study

Plasmid	Description; selectable marker	Source
pMV361	integrative *E. coli*/mycobacterial shuttle vector; Kan^R	Stover *et al.* 1991[30]
pSET152	*Streptomyces* vector with apramycin resistance gene (*aac(3)IV*), template for *apr* amplification; Apr^R	Wilkinson *et al.* 2002[29]
pGEM-T-easy	PCR saving vector; Amp^R	Promega
pMCS5	general cloning vector; Amp^R	MBio
pGEM-T-katG	intermediate vector for *katG* subcloning; Amp^R	this study
pMV361-*apr*	integrative *E. coli*/mycobacterial shuttle vector; Kan^R, Apr^R	this study
pMV361-*apr-katG*	derivative of pMV361-*apr* containing *M. tuberculosis katG*; Kan^R, Apr^R, INH^S	this study
pSE-*apr-katG*	derivative of pMV361-*apr-katG* deleted for mycobacteriophage integrase *int* and *attP*; intermediate vector used for cloning of Δ*aac(2′)*, Δ*eis1* and Δ*eis2* alleles; Kan^R, Apr^R, INH^S	this study
pMCS5-*MAB_4395*	intermediate vector containing *aac(2′)* flanking regions; Amp^R	
pSE-Δ*aac(2′)*	suicide vector; derivative of pSE-*apr-katG* carrying Δ*aac(2′)* allele; Apr^R, Kan^R, INH^S	this study
pSE-Δ*eis1*	suicide vector; derivative of pSE-*apr-katG* carrying Δ*eis1* allele; Apr^R, Kan^R, INH^S	this study
pSE-Δ*eis2*	suicide vector; derivative of pSE-*apr-katG* carrying Δ*eis2* allele; Apr^R, Kan^R, INH^S	this study
pMV361-*aac(2′)*	integrative complementation vector for *aac(2′)*, Kan^R	this study
pJB-*apr*	derivative of pMV361 in which the kanamycin resistance cassette was substituted by the apramycin resistance cassette; Apr^R	this study
pJB-*apr-eis2*	integrative complementation vector for *eis2*; derivative of pJB-*apr*; Apr^R	this study

Kan^R, kanamycin resistance cassette; Apr^R, apramycin resistance cassette; Amp^R, ampicillin resistance cassette; INH^S, isoniazid susceptibility cassette.

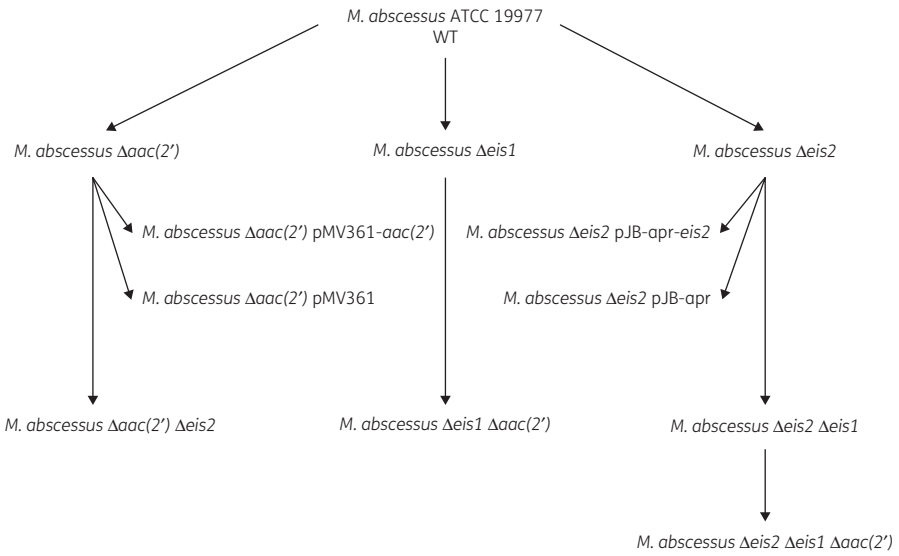


Figure 2. Genealogy of *M. abscessus* strains used in this study for the construction of single, double and triple deletion mutants.

relatively resistant to several AGs with a 2′-amino group. Amikacin is protected from AAC(2′) modification due to the presence of an OH group at position 2′. Actually, amikacin is a derivative of kanamycin A, but with an L-hydroxyaminobutyryl amide (L-HABA) side chain at position 1 of the 2-deoxystreptamine core. Tobramycin (3′-deoxy-kanamycin B) is inert to modification by 3′-phosphotransferases, but susceptible to modification by AAC(2′). This is indicated by the observation that the kanamycin B and tobramycin MICs are similarly decreased for the Δ*aac(2′)* mutant strain as compared with the parental strain. Since tobramycin is not a substrate for the 3′-AG-phosphotransferase [which is encoded on the backbone of the complementation vector pMV361-*aac(2′)*], restoration of the tobramycin phenotype could be addressed by transformation with the complementation vector. Phenotypic complementation was observed, indicating that deletion of *aac(2′)* is responsible for increased tobramycin susceptibility of the mutant. The MIC of arbekacin is similarly low for the WT strain and the Δ*aac(2′)* mutant and similar to the MIC of dibekacin for the Δ*aac(2′)* mutant, while the dibekacin MIC for the WT strain is much higher. Dibekacin (3′,4′-dideoxy-kanamycin B) differs from arbekacin at position 1 of

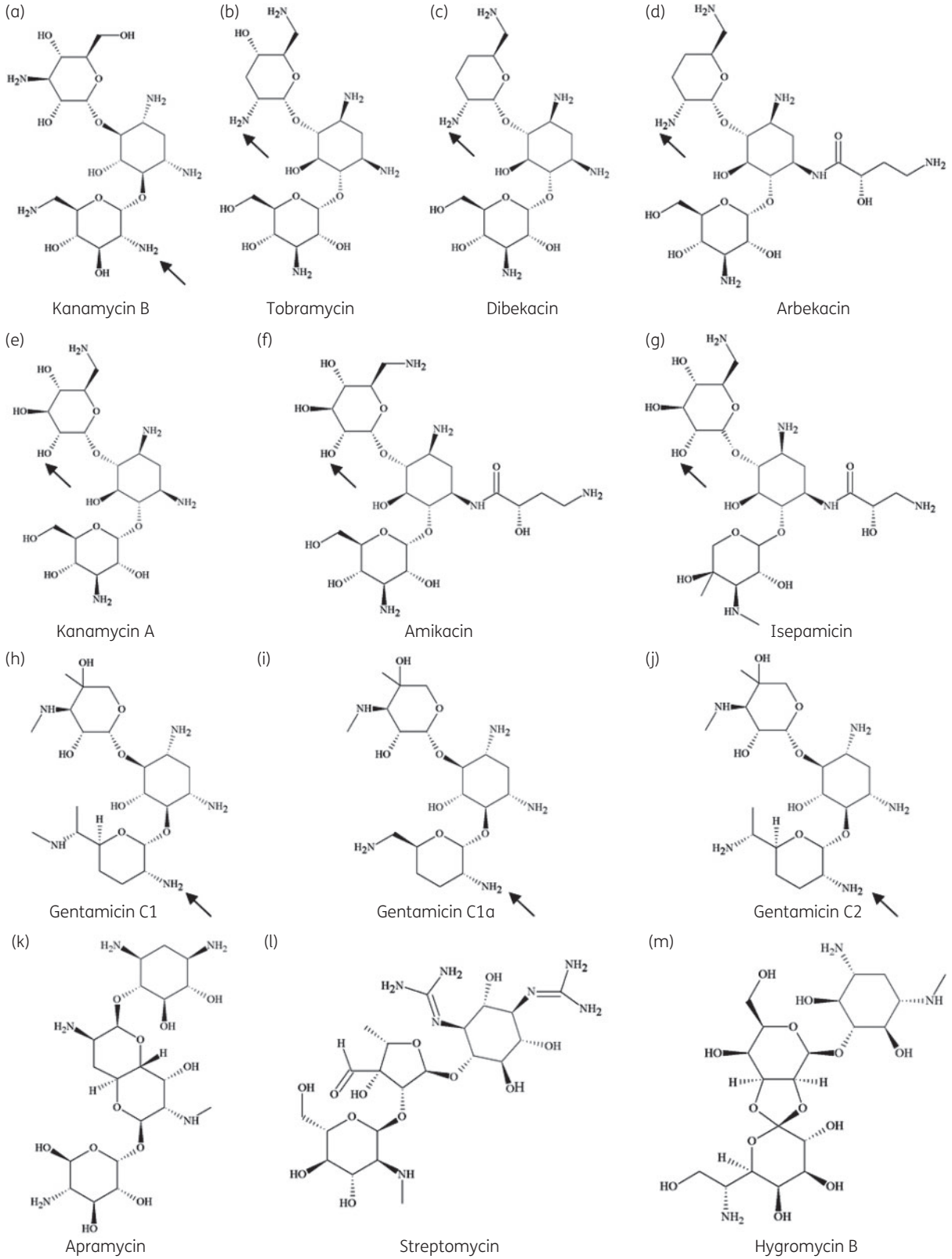


Figure 3. Structure of 4,6-disubstituted (a–j) and atypical (k–m) AGs used in this study. Black arrows show the 2′ groups of the 4,6-distubstituted AGs (a–j).

Table 3. MICs for *M. abscessus* deletion mutants (all values are medians, on day 5)[a,b]

	Strain							
Compound	WT	Δ*aac(2′)*	Δ*eis1*	Δ*eis2*	Δ*aac(2′)* Δ*eis1*	Δ*aac(2′)* Δ*eis2*	Δ*eis1* Δ*eis2*	Δ*aac(2′)* Δ*eis1* Δ*eis2*
Kanamycin B	8	0.125	8	2	0.125	0.125	1	0.125
Tobramycin	8	0.25	8	4	0.25	0.125	2	0.125
Dibekacin	16	1	16	16	2	1	16	1
Arbekacin	1	0.5	1	1	0.5	0.5	0.5	0.5
Gentamicin C	4	1	4	4	1	1	4	1
Isepamicin	1	1	1	0.5	1	0.5	0.5	0.5
Amikacin	2	2	2	0.25	2	0.25	0.25	0.25
Kanamycin A	1	1	1	0.5	1	0.5	0.5	0.25
Apramycin	0.5	0.5	1	0.5	0.5	0.5	0.5	0.5
Streptomycin	32	32	32	32	32	32	32	32
Hygromycin B	256	256	256	16	256	16	16	16
Capreomycin	128	128	256	4	128	4	4	4

[a]For a complete overview of MIC results from three independent experiments judged at days 3, 5, 7 and 12 see Table S2.
[b]For phenotype of single deletion mutants transformed with complementation vectors and control vectors also see Table S2.

the 2-deoxystreptamine core; there arbekacin carries an L-HABA chain, which obviously protects from AAC(2′) activity.

Gentamicin C is not a pure compound, but a mixture of different subclasses (C1, C2 and C1a). C-type gentamicin subgroups carry a 2′-NH_2 group. In contrast, B-type gentamicin carries a 2′-OH group. Isepamicin is a derivative of gentamicin B with an L-HABA side chain at position 1 of the 2-deoxystreptamine core. The MIC for the WT strain of isepamicin is lower than that of gentamicin C, while both compounds have similarly (low) MICs for the Δ*aac(2′)* mutant. These findings may be explained by the enzymatic modification of gentamicin C by the AAC(2′) activity present in the WT strain. Taken together, comparison of the MICs for the WT strain and the Δ*aac(2′)* mutant indicates that AAC(2′) confers resistance to drugs carrying a 2′-NH_2 group, unless position 1 of the 2-deoxystreptamine core is modified with an L-HABA chain (compare dibekacin and arbekacin). Neither deoxygenation at 3′ nor dideoxygenation at positions 3′ and 4′ (compare kanamycin B with tobramycin and dibekacin, respectively) prevent *M. abscessus* AAC(2′)-mediated drug resistance mechanisms. An approximately 10-fold decrease in the MICs of gentamicin C and tobramycin and an even higher decrease in the MIC of dibekacin were also seen upon genetic inactivation of *Mycobacterium smegmatis aac(2′)*.[36]

The *M. abscessus* Δ*eis1* mutant did not show any resistance phenotype and therefore does not contribute to AG resistance, at least not during *in vitro* growth. Expression studies and biochemical assays would be required to address the physiological and enzymatic function of Eis1. It may be hypothesized that promoter-up mutations might increase AG resistance. This hypothesis could also be addressed by transformation of WT strains with multi-copy vectors containing *eis1*. However besides AAC(2′)-mediated AG resistance, *M. abscessus* possesses at least a second mechanism of AG resistance, which is mediated by Eis2. The Δ*eis2* mutant showed increased susceptibility to the peptide antibiotic capreomycin and to a distinct and more heterogeneous group of AGs: kanamycin B, amikacin and hygromycin B. Eis proteins of different origin have been shown to have multiple AG- and capreomycin-acetylating activities, e.g. *M. tuberculosis* and *M. smegmatis* Eis tri-acetylate neamine in a sequential manner, first at position 2′, then at position 6′ and finally at position 1. The number of acetylations depends on the AG itself, but also on the biological origin of the enzyme. Up to four acetyl residues may be transferred to tobramycin by *M. tuberculosis* Eis and *M. smegmatis* Eis, respectively.[33,37,38] Hygromycin is mono- and di-acetylated by *M. smegmatis* and *M. tuberculosis* Eis, respectively. Apramycin is diacetylated by *M. smegmatis*, but is not a substrate for *M. tuberculosis* Eis. *M. abscessus* Eis2 does not inactivate apramycin (identical MICs for the WT strain and the Δ*eis2* mutant), although this does not exclude that apramycin is an *M. abscessus* Eis2 substrate. Corresponding acetylations might just not affect antibacterial activity. A combined application of an AAC(2′) inhibitor together with specific NH_2-AGs (e.g. kanamycin B and gentamicin C) could enhance the activity of these AGs. Administration of an Eis2 inhibitor could enhance the activity of capreomycin and amikacin, the latter being a cornerstone for treatment of *M. abscessus* infections.[39] As opposed to several 2-deoxystreptamine AGs, streptomycin MICs for the mutant strains remained at the high WT MIC level, pointing to a distinct streptomycin resistance mechanism in *M. abscessus*. Our study demonstrates that apramycin, an AG with little ototoxicity,[40] arbekacin, isepamicin and kanamycin A exhibit excellent *in vitro* activities against the *M. abscessus* ATCC 19977 type strain and that the activity of these drugs is not affected by AAC(2′) and Eis proteins, respectively. Our data further support MIC determination of these AGs for a broader set of *M. abscessus* clinical isolates.[15] The results from the suggested *in vitro* studies may provide a rational basis for designing clinical trials aiming at implementation of improved treatment regimens against one of the most drug-resistant pathogens, *M. abscessus*.

Acknowledgements

We thank M. I. Hutchings for plasmid pSET152 and E. C. Böttger for helpful discussion.

Funding

This study was supported by a grant from the Swiss National Science Foundation (no. 31003 A_153349). The Institute of Medical Microbiology and the University of Zurich continuously supported this study.

Transparency declarations

None to declare.

Supplementary data

Tables S1 and S2 are available as Supplementary data at *JAC* Online.

References

1 Esther CR Jr, Esserman DA, Gilligan P *et al*. Chronic *Mycobacterium abscessus* infection and lung function decline in cystic fibrosis. *J Cyst Fibros* 2010; **9**: 117–23.

2 Griffith DE. Nontuberculous mycobacterial lung disease. *Curr Opin Infect Dis* 2010; **23**: 185–90.

3 Furuya EY, Paez A, Srinivasan A *et al*. Outbreak of *Mycobacterium abscessus* wound infections among "lipotourists" from the United States who underwent abdominoplasty in the Dominican Republic. *Clin Infect Dis* 2008; **46**: 1181–8.

4 Maurer F, Castelberg C, von Braun A *et al*. Postsurgical wound infections due to rapidly growing mycobacteria in Swiss medical tourists following cosmetic surgery in Latin America between 2012 and 2014. *Euro Surveill* 2014; **19**: pii=20905.

5 Newman MI, Camberos AE, Ascherman J. *Mycobacteria abscessus* outbreak in US patients linked to offshore surgicenter. *Ann Plast Surg* 2005; **55**: 107–10; discussion 110.

6 Ricciardo B, Weedon D, Butler G. *Mycobacterium abscessus* infection complicating a professional tattoo. *Australas J Dermatol* 2010; **51**: 287–9.

7 Taylor JL, Palmer SM. *Mycobacterium abscessus* chest wall and pulmonary infection in a cystic fibrosis lung transplant recipient. *J Heart Lung Transplant* 2006; **25**: 985–8.

8 Viana-Niero C, Lima KV, Lopes ML *et al*. Molecular characterization of *Mycobacterium massiliense* and *Mycobacterium bolletii* in isolates collected from outbreaks of infections after laparoscopic surgeries and cosmetic procedures. *J Clin Microbiol* 2008; **46**: 850–5.

9 Brown-Elliott BA, Nash KA, Wallace RJ Jr. Antimicrobial susceptibility testing, drug resistance mechanisms, and therapy of infections with nontuberculous mycobacteria. *Clin Microbiol Rev* 2012; **25**: 545–82.

10 Nessar R, Cambau E, Reyrat JM *et al*. *Mycobacterium abscessus*: a new antibiotic nightmare. *J Antimicrob Chemother* 2012; **67**: 810–8.

11 Griffith DE. Therapy of nontuberculous mycobacterial disease. *Curr Opin Infect Dis* 2007; **20**: 198–203.

12 Prammananan T, Sander P, Brown BA *et al*. A single 16S ribosomal RNA substitution is responsible for resistance to amikacin and other 2-deoxystreptamine aminoglycosides in *Mycobacterium abscessus* and *Mycobacterium chelonae*. *J Infect Dis* 1998; **177**: 1573–81.

13 Sander P, Böttger EC. Mycobacteria: genetics of resistance and implications for treatment. *Chemotherapy* 1999; **45**: 95–108.

14 Wallace RJ, Meier A, Brown BA *et al*. Genetic basis for clarithromycin resistance among isolates of *Mycobacterium chelonae* and *Mycobacterium abscessus*. *Antimicrob Agents Chemother* 1996; **40**: 1676–81.

15 Maurer FP, Bruderer VL, Castelberg C *et al*. Aminoglycoside-modifying enzymes determine the innate susceptibility to aminoglycoside antibiotics in rapidly growing mycobacteria. *J Antimicrob Chemother* 2015; **70**: 1412–9.

16 Magnet S, Blanchard JS. Molecular insights into aminoglycoside action and resistance. *Chem Rev* 2005; **105**: 477–98.

17 Ripoll F, Pasek S, Schenowitz C *et al*. Non-mycobacterial virulence genes in the genome of the emerging pathogen *Mycobacterium abscessus*. *PLoS One* 2009; **4**: e5660.

18 Lamrabet O, Drancourt M. Genetic engineering of *Mycobacterium tuberculosis*: a review. *Tuberculosis (Edinb)* 2012; **92**: 365–76.

19 Cortes M, Singh AK, Reyrat JM *et al*. Conditional gene expression in *Mycobacterium abscessus*. *PLoS One* 2011; **6**: e29306.

20 Medjahed H, Reyrat JM. Construction of *Mycobacterium abscessus* defined glycopeptidolipid mutants: comparison of genetic tools. *Appl Environ Microbiol* 2009; **75**: 1331–8.

21 Pawlik A, Garnier G, Orgeur M *et al*. Identification and characterization of the genetic changes responsible for the characteristic smooth-to-rough morphotype alterations of clinically persistent *Mycobacterium abscessus*. *Mol Microbiol* 2013; **90**: 612–29.

22 Halloum I, Carrère-Kremer S, Blaise M *et al*. Deletion of a dehydratase important for intracellular growth and cording renders rough *Mycobacterium abscessus* avirulent. *Proc Natl Acad Sci USA* 2016; **113**: E4228–37.

23 Kim YS, Yang CS, Nguyen LT *et al*. *Mycobacterium abscessus* ESX-3 plays an important role in host inflammatory and pathological responses during infection. *Microbes Infect* 2017; **19**: 5–17.

24 Lefebvre AL, Dubée V, Cortes M *et al*. Bactericidal and intracellular activity of β-lactams against *Mycobacterium abscessus*. *J Antimicrob Chemother* 2016; **71**: 1556–63.

25 Viljoen A, Blaise M, de Chastellier C *et al*. *MAB_3551c* encodes the primary triacylglycerol synthase involved in lipid accumulation in *Mycobacterium abscessus*. *Mol Microbiol* 2016; **102**: 611–27.

26 Rominski A, Roditscheff A, Selchow P *et al*. Intrinsic rifamycin resistance of *Mycobacterium abscessus* is mediated by ADP-ribosyltransferase MAB_0591. *J Antimicrob Chemother* 2017; **72**: 376–84.

27 Sander P, Papavinasasundaram KG, Dick T *et al*. *Mycobacterium bovis* BCG *recA* deletion mutant shows increased susceptibility to DNA-damaging agents but wild-type survival in a mouse infection model. *Infect Immun* 2001; **69**: 3562–8.

28 Sander P, Meier A, Böttger EC. *rpsL*$^{+}$: a dominant selectable marker for gene replacement in mycobacteria. *Mol Microbiol* 1995; **16**: 991–1000.

29 Wilkinson CJ, Hughes-Thomas ZA, Martin CJ *et al*. Increasing the efficiency of heterologous promoters in actinomycetes. *J Mol Microbiol Biotechnol* 2002; **4**: 417–26.

30 Stover CK, de la Cruz VF, Fuerst TR *et al*. New use of BCG for recombinant vaccines. *Nature* 1991; **351**: 456–60.

31 Clinical and Laboratory Standards Institute. *Susceptibility Testing of Mycobacteria, Nocardiae, and Other Aerobic Actinomycetes—Second Edition: Approved Standard M24-A2*. CLSI, Wayne, PA, USA, 2011.

32 Garneau-Tsodikova S, Labby KJ. Mechanisms of resistance to aminoglycoside antibiotics: overview and perspectives. *Medchemcomm* 2016; **7**: 11–27.

33 Pricer RE, Houghton JL, Green KD *et al*. Biochemical and structural analysis of aminoglycoside acetyltransferase Eis from *Anabaena variabilis*. *Mol Biosyst* 2012; **8**: 3305–13.

34 Zaunbrecher MA, Sikes RD Jr, Metchock B *et al*. Overexpression of the chromosomally encoded aminoglycoside acetyltransferase *eis* confers kanamycin resistance in *Mycobacterium tuberculosis*. *Proc Natl Acad Sci USA* 2009; **106**: 20004–9.

35 Heym B, Zhang Y, Poulet S *et al*. Characterization of the *katG* gene encoding a catalase-peroxidase required for the isoniazid susceptibility of *Mycobacterium tuberculosis*. *J Bacteriol* 1993; **175**: 4255–9.

36 Ainsa JA, Perez E, Pelicic V *et al*. Aminoglycoside 2′-*N*-acetyltransferase genes are universally present in mycobacteria: characterization of the *aac(2′)-Ic* gene from *Mycobacterium tuberculosis* and the *aac(2′)-Id* gene from *Mycobacterium smegmatis*. *Mol Microbiol* 1997; **24**: 431–41.

37 Chen W, Green KD, Tsodikov OV *et al*. Aminoglycoside multiacetylating activity of the enhanced intracellular survival protein from *Mycobacterium smegmatis* and its inhibition. *Biochemistry* 2012; **51**: 4959–67.

38 Houghton JL, Green KD, Pricer RE *et al*. Unexpected *N*-acetylation of capreomycin by mycobacterial Eis enzymes. *J Antimicrob Chemother* 2013; **68**: 800–5.

39 Willby MJ, Green KD, Gajadeera CS *et al*. Potent inhibitors of acetyltransferase Eis overcome kanamycin resistance in *Mycobacterium tuberculosis*. *ACS Chem Biol* 2016; **11**: 1639–46.

40 Matt T, Ng CL, Lang K *et al*. Dissociation of antibacterial activity and aminoglycoside ototoxicity in the 4-monosubstituted 2-deoxystreptamine apramycin. *Proc Natl Acad Sci USA* 2012; **109**: 10984–9.